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# Phorbol ester inhibits phenobarbital induction of cytochrome P-450.

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Phorbol Ester Inhibits Phenobarbital Induction of  
Cytochrome P-450

by

David Francis Steele

A Thesis  
submitted to the  
Faculty of Graduate Studies and Research  
through the Department of  
Biological Sciences in Partial Fulfillment  
of the Requirements for the Degree  
of Master of Science at  
the University of Windsor

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1987

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# ABSTRACT

## Phorbol Ester Inhibits Phenobarbital Induction of Cytochrome P-450

by

David Francis Steele

The mechanism by which phenobarbital induces cytochrome P-450 in the liver is unknown. We have employed the phorbol ester, 12-o-tetradecanoylphorbol-13-acetate (TPA), in an attempt to determine whether protein kinase C plays a role in this process. In hypophysectomized male rats, TPA significantly lowered basal levels of cytochrome P-450, aniline hydroxylase activity, and ethylmorphine demethylase activity, and also impeded the induction of these parameters by phenobarbital. TPA did not affect basal cytochrome c reductase activity, but impeded its induction by phenobarbital. TPA neither bound nor directly inhibited cytochrome P-450. Instead, the effects of TPA were apparently due to a decrease in the levels of apocytochrome P-450, since incubation of liver homogenates with hemin did not increase microsomal holocytochrome P-450 content. TPA significantly attenuated the increase in hepatic RNA promoted by phenobarbital. It is likely, therefore, that TPA

interferes with induction at a pretranslational level.

Puromycin blocked the effects of TPA, suggesting that the TPA effects are mediated by a peptide. Induction of cytochrome P-450 by 3-methylcholanthrene was also impeded by TPA. The effects of TPA may, therefore, be general, applying to several or perhaps all classes of inducer. In any case, these data are consistent with a role for kinase C in P-450 regulation. A model is presented to account for these findings and others from the literature.

For my family.



## ACKNOWLEDGEMENTS

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## INTRODUCTION

The cytochromes P-450 are a ubiquitous group of mono-oxygenases active in the metabolism of lipophilic compounds both endogenous and exogenous. These enzymes are hemoproteins found imbedded in the endoplasmic reticulum and inner mitochondrial membrane of all eukaryotes (Stryer, 1981). All function to insert an oxygen atom into hydrophobic compounds, thus increasing their hydrophilicity. Numerous nonenzymatic rearrangements may also be thus promoted (Astrom and DePierre, 1986; Conney, 1986; Holland, 1982).

In order to function, all cytochromes P-450 require an electron-donating enzyme as well as molecular oxygen. In the mitochondrion, this enzyme is ferredoxin, and is itself reduced by ferredoxin reductase, the electrons being derived from NADPH by this latter enzyme. In the endoplasmic reticulum, a different enzyme, capable of directly transferring electrons from NADPH to cytochrome P-450 is functional. This enzyme is a flavoprotein known as cytochrome P-450 reductase (Figure One). It is often assayed as cytochrome c reductase with which it is probably identical.

The cytochromes P-450 are thought to operate as



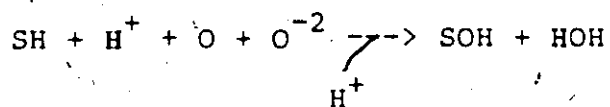
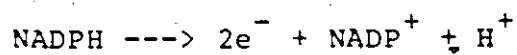


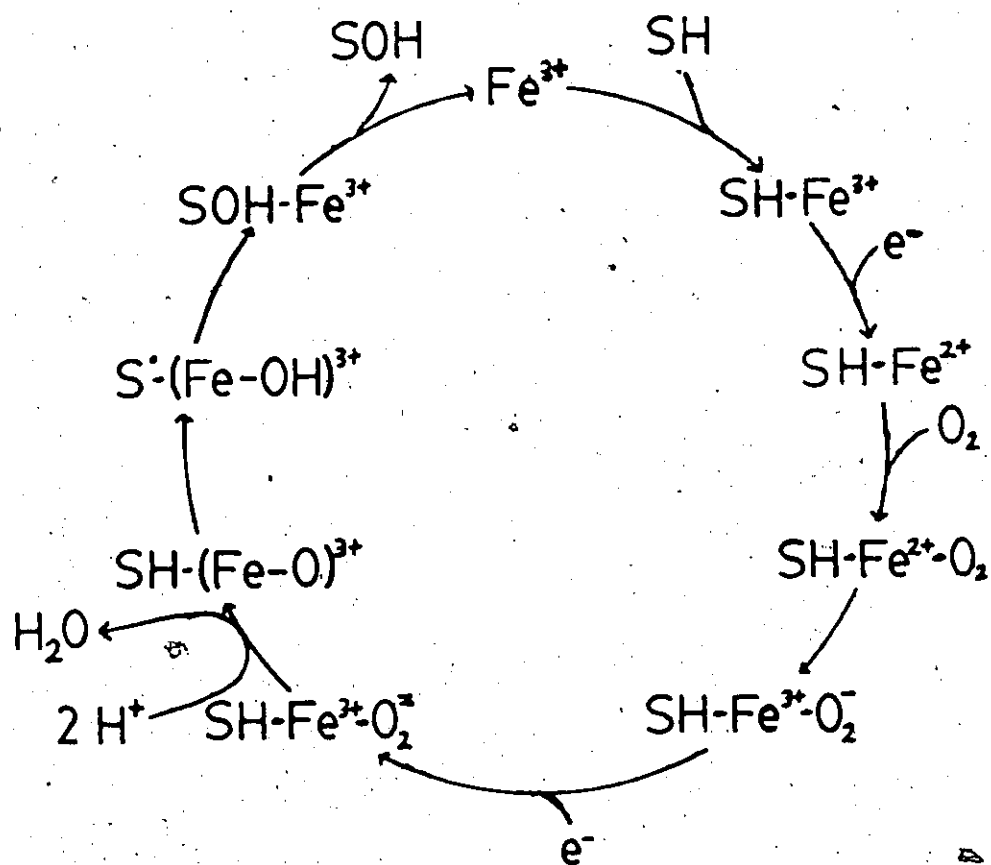
FIGURE ONE. Electron transfer in the microsomal cytochrome P-450 system. Electrons are transferred from NADPH to cytochrome P-450 by cytochrome P-450 reductase. The electrons are transferred by the cytochrome to molecular oxygen which then reacts with substrate (SH) and 2 H<sup>+</sup> yielding hydroxylated substrate (SOH) and water.

illustrated in Figure Two. Substrate binds to the cytochrome when the heme iron is in its oxidized (+3) state. At this point, an electron is donated by the reductase converting the iron to the +2 state. Molecular oxygen now binds also to the cytochrome, and a second electron is supplied by the reductase (or, in some cases, from NADH via cytochrome b5). The oxygen-oxygen bond is then split yielding water and, by hydrogen abstraction followed by radical recombination (Holland, 1982), a hydroxylated substrate as well as reoxidized heme iron. Following dissociation of the product, the cycle is free to repeat.

Many isozymes of cytochrome P-450 are, under most conditions, present only at very low levels. The levels of some of these isozymes are greatly increased in the presence of molecules which serve as their substrates. These isozymes are considered "inducible". There are several classes of such inducible enzymes. Some are inducible by polycyclic aromatic hydrocarbons, others by specific steroids, and still others by compounds such as phenobarbital.

The mechanism of induction of the isozymes whose synthesis is promoted by the polycyclic aromatic hydrocarbons is best understood. These isozymes are found in most tissues of the body, and are induced via a system resembling that functional for most steroid actions. According to the

FIGURE TWO. Cytochrome P-450 reaction cycle. Substrate (S-H) binds to P-450 when the heme iron is in the +3 state. An electron is then donated by the reductase, converting the iron to the +2 state. Oxygen next binds to the cytochrome, and a second electron is provided by the reductase. The oxygen-oxygen bond is split yielding water and hydroxylated substrate (S-OH).



most widely accepted model, the polycyclic aromatic hydrocarbon enters the cell where it binds to a cytoplasmic receptor analogous to those for glucocorticoids or sex steroids. Some would argue that this receptor binding is nuclear, but whatever the locale of binding, the model remains essentially the same.

Following ligand binding, the receptor translocates to the nucleus and binds to the chromatin so as to activate the transcription of various genes including those encoding the polycyclic aromatic hydrocarbon-inducible isozymes of cytochrome P-450. These isozymes, and other metabolic enzymes coinduced with them, then metabolize the inducing compound, decreasing its hydrophobicity, and, of course, acting to remove the inducing signal. Loss of hydrophobicity is prerequisite to the excretion of the compound.

Other classes of inducible isozymes are confined to the liver. One of these classes is that inducible by steroids typified by pregnenolone carbonitrile. A particularly interesting feature of this induction process is its apparent independence from classical steroid receptors (Hardwick, Gonzalez and Kasper, 1983; Heuman, et al., 1982; Gonzalez, et al., 1985). The mechanism(s) by which these compounds exert their inductive effects are not known.

Phenobarbital is the prototype of another class of

compounds that induce the synthesis of a different group of specific isozymes of hepatic cytochrome P-450 as well as a number of other hepatic enzymes. Again, through mechanisms unknown. In fact, phenobarbital was the first drug noted to have inductive effects. Attempts to find a receptor such as that for the polycyclic aromatic hydrocarbons have met with no success. To date, no receptor has been found.

Administration of phenobarbital to the rat results in increased levels of hepatic cytochrome P-450, mainly as a result of increased transcription of genes encoding the specific isozymes b and e (Rees, 1979; Hardwick, Schwalm, and Richardson, 1983). Other effects associated with induction include reduced turnover of hepatic messenger and ribosomal RNAs (Lechner and Pousada, 1971), and increased translation of P-450-encoding mRNAs (Dubois and Waterman, 1979; Gozukara, et al., 1984). Translational rates in general are increased (Venkatesan and Steele, 1975), and a proliferation of smooth endoplasmic reticulum is seen (Ernster and Orrenius, 1965; Orrenius, Ericsson, and Ernster, 1965). There is an enhancement of protein per unit weight of liver tissue (Conney and Gilman, 1963), and the activity of a number of enzymes is increased. Among these are NADPH-cytochrome c reductase (Rees, 1979), ornithine decarboxylase (Van Rooijen, et al., 1984),<sup>0</sup> UDP-glucuronyltransferase

(Wishart, 1978),  $\phi$ -aldehyde dehydrogenase (Deitrich, et al., 1978), microsomal epoxide hydrolase (Oesch, Jerina, and Daly, 1971), and glutathione-S-transferase (Kaplowitz, Kuhlenskamp, and Clifton, 1975). Yet, as stated above, a receptor for phenobarbital has yet to be found (Rees, 1979; Hardwick, Schwalm, and Richardson, 1983).

One possibility for the mechanism by which phenobarbital induces hepatic cytochromes P-450 has recently been eliminated. It had been argued that the inducible cytochromes P-450 themselves might serve as the phenobarbital receptor. Specifically, it was argued that an endogenous signal molecule was normally metabolized by these cytochromes. When the metabolism of this substance was impeded by the presence of phenobarbital, the induction response would be triggered.

Ortiz de Montellano and Costa (1986) suppressed the catalytic activities of the phenobarbital-inducible P-450 isozymes with an irreversible inhibitor, 1-aminobenzotriazole. This compound not only failed to induce the same (of any other) cytochromes P-450, but also failed, at the level of transcription, to interfere with their induction by phenobarbital. It seems highly unlikely, therefore, that phenobarbital-mediated induction is related directly to the catalytic activities of the metabolizing P-450s. Other avenues must be explored.

Phenobarbital is known to depress neurological activity. This is achieved by interacting with neuronal plasma membrane (Goldberg, 1980; Maynert and Kusek, 1980; Novak and Swift, 1976; Vilallonga and Phillips, 1980). It seems possible that phenobarbital exerts its hepatic effects via similar interactions. If this is true, then there must be a means by which the perturbations in membrane structure caused by phenobarbital are translated into signals that can affect gene activity. One group of enzymes known to be both profoundly affected by membrane changes and capable of eliciting changes in gene expression are the calcium / phospholipid-dependent protein kinases known collectively as protein kinase C (Kuo, et al., 1986; Housey, et al., 1987; Ohno, et al., 1987). For the purposes of this discussion, these enzymes shall be considered as one.

Protein kinase C exists, in the unstimulated state, as an inactive, water-soluble cytosolic enzyme (Takai, et al., 1985). When the physiological activator of this enzyme, diacylglycerol, is present in the plasma membrane, the kinase translocates to that structure and becomes active (Minakuchi, et al., 1981).

Diacylglycerol is normally a product of signal-induced turnover of membrane phosphatidylinositol (Minakuchi, et al., 1981; Kuo, et al., 1986). The presence of diacylglycerol in



the plasma membrane causes an increase in the spacing of phospholipid polar groups (Das and Rand, 1986). This change, combined with a probable affinity of the kinase for the diacylglycerol itself (Hannun, Loomis, and Bell, 1985, 1986), promotes the binding of a specific domain (Mouchly-Rosen and Koshland, 1987; Lee and Bell, 1986) to the membrane. This binding directly activates the kinase (Nishizuka, 1984).

Another component of the membrane necessary to this process is phosphatidylserine (Nishizuka, et al., 1984; Kuo, et al., 1980). Many compounds with high affinities for this phospholipid are inhibitors of kinase C activation (Mori, et al., 1980). Some P-450 inducers, such as chlorpromazine and imipramine (Parke, 1975) fall into this category (Mori, et al., 1980). Phenobarbital itself binds to phosphatidylcholine, sphingomyelin, and cardiolipin (Maynert and Kusek, 1980; Novak and Swift, 1976), but does not interact measurably with phosphatidylserine (Novak and Swift, 1976).

That phenobarbital does not bind phosphatidylserine does not necessarily mean that phenobarbital does not act similarly to the other membrane-active inducers of cytochrome P-450. Kinase C activation does require perturbations in membrane structure as introduced by diacylglycerol or synthetic activators. It is quite plausible that mechanisms other than requiring phosphatidylserine could reduce kinase

C binding / activation. This argument is complemented by the facts that several structurally unrelated compounds are capable of activating the kinase, apparently as a result of their interactions with membrane (Nishizuka, et al., 1984), and that numerous substrates of kinase C exist which do not require the phospholipid for phosphorylation by the kinase (Bazzi and Nelsestuen, 1987).

Phenobarbital might also act to ~~inhibit~~ inhibit kinase C directly. Chauhan and Brockerhoff (1987), have recently reported just such an inhibitory action by phenobarbital. According to these workers, the drug competes with diacylglycerol for the activating site on the kinase. They synthesized their own phenobarbital, however. Thus one cannot be absolutely certain that their results reflect a true effect of the drug.

The fact that all kinase C cDNAs sequenced to date contain a domain encoding "zinc fingers" (Ohno, et al., 1987), when combined with the finding that phenobarbital directly binds to kinase C, suggests yet another possibility. Zinc fingers are DNA binding domains, and have been found encoded also in the DNA sequences for glucocorticoid and estradiol receptors (Giguere, et al., 1986; Greene, et al., 1986). The homology of this kinase C domain to those of the steroid receptors is quite marked (Ohno, et al., 1987). Perhaps the

binding of phenobarbital to kinase C could do more than simply inhibit the activation of the enzyme. Perhaps kinase C is a nuclear-translocating receptor for phenobarbital.

If phenobarbital exerts any of its hepatic effects through some sort of inhibitory action with protein kinase C, then activation of the kinase should oppose these effects. 12-O-Tetradecanoylphorbol-13-acetate (TPA) is a potent and direct activator of kinase C (Castagna, et al., 1982; Niedel, Kuhn, and Vandenbark, 1983), functioning as a long-lived diacylglycerol analog (Lee and Bell, 1986; Nishizuka, 1984). We have employed this activator in concert with phenobarbital and other drugs in an attempt to gain insight into the mode of action of phenobarbital in inducing liver cytochromes P-450.

## MATERIALS AND METHODS

### Chemicals

12-O-Tetradecanoylphorbol-13-acetate, 3-methylcholanthrene, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, Trizma base, and chlorpromazine hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO. Sodium phenobarbital was a gift from E.V. Wilson, Bureau of Dangerous Drugs, Health Protection Branch, Ottawa, ON. Acetylacetone, ammonium acetate, barium hydroxide, cupric sulfate pentahydrate, ethanol, HCl, methanol, phenol crystals, semicarbazide, sodium carbonate, sodium chloride, sodium hydroxide, mono- and dibasic sodium phosphate, sodium tartrate, and zinc sulfate were obtained from Fisher Scientific Co., Toronto, ON. BDH Chemicals (Toronto, ON) supplied ethylmorphine hydrochloride. Magnesium chloride and potassium chloride were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ. Liquified phenol was obtained from Fluka Chemical Corp., Hauppesburg, NY. All other chemicals were of reagent grade or better.

## Animals

Male Wistar rats (175-200g) (Charles River Canada, Inc., St. Constant, PQ) were employed in all experiments. Purina Lab Chow and tap water were provided ad libitum. Hypophysectomized rats (surgery performed by Charles River) were supplied with a solution of 5% (w/v) dextrose/0.9% NaCl as drinking water. The rats were housed in steel mesh cages suspended over absorbent paper and hardwood shavings. A constant 12 hour photoperiod (lights on at 7:00 a.m.) was maintained, and the temperature was maintained at 24C.

## Drug Treatments

All drugs were injected via the intraperitoneal route: 12-O-tetradecanoylphorbol-13-acetate was given in 0.5 mL/kg body weight. Phenobarbital in 1.0 mL/kg body weight, 3-methylcholanthrene in 1.0 mL/kg body weight, and chlorpromazine hydrochloride in 1.0 mL/kg body weight. The doses

are specified in Results. TPA was administered in 0.16% DMSO, 70% 0.15M NaCl, 30% ethanol. Phenobarbital was also administered in this vehicle. 3-Methylcholanthrene was administered in corn oil; chlorpromazine, in 0.9% saline.

#### Preparation of Microsomes

Rats were weighed, decapitated, and exsanguinated. The liver was rapidly excised and placed in ice-cold homogenization buffer (50 mM TrisHCl, pH 7.4, containing 150 mM KCl). All subsequent operations were performed at 4°C. The liver was rinsed in the buffer, blotted dry, and weighed. It was then minced on an ice-chilled diffusion glass and homogenized in 3 volumes of fresh homogenization buffer using a Polytron PT20 homogenizer (Brinkmann Instruments, Toronto, ON). The homogenate was centrifuged for 20 minutes at 9000g in a Beckman J-21C refrigerated centrifuge (JA 21 rotor). 10 mL aliquots of the supernatant were withdrawn from below the fat layer and centrifuged for 60 minutes at 105,000g in a Beckman L5-65 ultracentrifuge (Ti 60 rotor).

The resulting pellets were rinsed with 5 mL of homogenization buffer and resuspended in 10 mL of the buffer by use of the Polytron. After centrifugation for 60 minutes at 105,000g, the microsomal pellets were rinsed with 50 mM Tris-HCl (pH 7.4) and suspended, by use of the Polytron, in 5mL of the same buffer. Protein concentration was determined by the method of Lowry, et al. (1951), using, as a standard, bovine serum albumin in 50 mM Tris-HCl (pH 7.4) to correct for Tris interference.

#### Cytochrome P-450 Quantitation

Cytochrome P-450 was quantified by the spectrum of (reduced microsomes plus carbon monoxide) minus (reduced microsomes) method. The microsomal suspension was diluted with 50 mM TrisHCl (pH 7.4) to a protein concentration of 1 mg/mL. It was allowed to warm to room temperature, then was reduced with a small quantity of sodium dithionite. The reduced microsomes were divided into two cuvettes, reference and sample, and a baseline difference spectrum (sample minus

reference) was measured (Perkin-Elmer 575 double beam spectrophotometer) over the wavelengths from 400 to 500 nm. Carbon monoxide was then gently bubbled for 30 seconds through the suspension in the sample cuvette. A difference spectrum was again recorded over 400-500 nm. P-450 concentration was calculated using the extinction coefficient ( $E_{450-490}$ ) of 91 per mM per cm (Omura and Sato, 1964).

#### NADPH-cytochrome c Reductase Activity

NADPH-cytochrome c reductase activity was assayed by the method of Langdon and Phillips (1962). The reaction mixture in both sample and reference cuvettes consisted of 50  $\mu$ M cytochrome c, 0.33  $\mu$ M KCN, and 90  $\mu$ g of microsomal protein in 2.9 mL of 33 mM phosphate buffer (pH 7.6). To the reference cuvette was added 0.1 mL of the phosphate buffer. The sample cuvette received 133  $\mu$ moles of NADPH in 0.1 mL of the same buffer. The rate of change of absorbance at 550 nm in the sample relative to the reference was used to determine the rate of cytochrome c reduction. An extinction coef-



ficient (E 550) of 19.6 per mM per cm for cytochrome c reduction was employed to calculate the nmols of cytochrome c reduced. The specific activity of the reductase was estimated by calculating the nmols reduced / mg microsomal protein / minute.

#### Ethylmorphine Demethylase Assay

N-Demethylation of ethylmorphine was measured by the method of Cochin and Axelrod (1959). This method assays the formaldehyde produced in the demethylatin reaction. Microsomal protein (2 mg/mL) was incubated at 37C, for 30 minutes, under air, in a Dubnoff shaking incubator (GCA Corp., Chicago, IL) in the presence of an NADPH generating system: 1.0 mM NADP, 2 units of glucose-6-phosphate dehydrogenase, 3.3 mM glucose-6-phosphate, and 8.3 mM MgCl. Also present in the incubates was 1.0 mM semicarbazide and 6.7 mM ethylmorphine hydrochloride, in 50 mM Tris HCl (pH 7.4) (total volume = 3.0 mL). A blank, identical to the sample except that ethylmorphine hydrochloride was excluded, was run for

each microsomal sample. The incubate, minus microsomal protein, was agitated in the incubator for 5 minutes prior to the addition of microsomes. At the end of the 30 minute incubation with the microsomes, the reaction was stopped by the addition of 5 mL of 8% zinc sulfate and 2 mL of saturated barium hydroxide. The sample was centrifuged for 10 minutes in an IEC Model K centrifuge at setting 40. A 5 mL aliquot of the resultant supernatant was added to 2.0 mL of double strength Nash reagent. The samples were then heated in a water bath at 60C for 30 minutes. Absorbance at 415 nm was read immediately against distilled water in a Perkin-Elmer 575 double beam spectrophotometer. The concentration of formaldehyde in the mix was estimated from a standard curve, and the specific activity was estimated by calculating the product formed in nmols / mg microsomal protein / minute.

#### Aniline Hydroxylase Assay

Formation of p-aminophenol from aniline was measured by

the method of Imai, Ito, and Sato (1966). The microsomal protein (2 mg / mL) was incubated in the presence of an NADPH generating system, under the same conditions used in the ethylmorphine assay (less semicarbazide), for 30 minutes in the presence of 4 mM aniline. At the end of the incubation period, the incubate was added to 1.5 mL of 20% (w/v) aqueous trichloroacetic acid and centrifuged for 10 minutes at setting 40 in an IEC Model K centrifuge. A 3.0 mL aliquot of the resulting supernatant was transferred to a test tube containing 1.5 mL of 10% (w/v) aqueous sodium carbonate and 3.0 mL of 2% (w/v) phenol in 0.2 N NaOH. A blank, 3.0 mL of 6.67% (w/v) trichloroacetic acid, was treated similarly. The solution was allowed to sit at room temperature for 30 minutes prior to the measurement of its absorbance at 630 nm (Perkin-Elmer 575 double beam spectrophotometer). The concentration of p-aminophenol was estimated with the use of a standard curve and the specific activity (nmols product formed / mg microsomal protein / minute) of the enzyme was estimated.

V

## Reconstitution of Holocytochrome P-450 by Hemin

Apocytochrome P-450 was estimated by the method of Correia and Meyer (1975). Hemin was dissolved in 2.0 mL of 0.1 M NaOH and the pH was then adjusted to 7.4 with 2.8 mL of 0.1 M Na-K phosphate buffer, pH 5.7. The liver was homogenized in 2 volumes of 50 mM Tris HCl, containing 150 mM KCl (pH 7.4), and divided into 2 equal aliquots. Each aliquot was transferred to a 25 mL Erlenmeyer flask containing 1 mM phosphatidylcholine and 0.25 mM phosphatidylethanolamine (final concentration). One of the aliquots was incubated with 40  $\mu$ M hemin for 20 minutes at 37°C in a Dubnoff shaking incubator. Buffer carrier substituted for hemin in the second flask. At the end of the incubation period, microsomes were prepared in the usual way, and cytochrome P-450 was assayed. Apocytochrome P-450 present was estimated by subtracting the estimated P-450 concentration of the aliquot incubated in the absence of hemin from the estimated P-450 concentration of the aliquot incubated with the hemin.

## Isolation and Quantitation of Hepatic RNA

The method described by Kruh (1967) was employed to isolate RNA. All glassware was heated at 200°C for at least 4 hours, plasticware was autoclaved, and water was treated with diethylpyrocarbonate prior to use. Liver was minced then homogenized, on ice, in 4 volumes of phenol by use of the Polytron. Two volumes of water were added and the mixture was stirred at room temperature for one hour. It was then centrifuged for 30 minutes at 5,000g in a Beckman J-21C centrifuge (JA 21 rotor) at 4°C. The resulting supernatant was removed and stored on ice and the phenolic phase was extracted with a volume of water equal to that of the supernatant removed. Following centrifugation at 5000g for 30 minutes, the supernatant was removed and pooled with the first aqueous extract. An equal volume of phenol was added to the pooled supernatants, and the suspension was centrifuged as above. To the final supernatant was added 0.1 volume of 20% potassium acetate and 2.5 volumes of ice cold 95% ethanol. The RNA was allowed to precipitate overnight at -20°C. The precipitated RNA was collected by centrifugation (5000g for 20 minutes at 4°C). The pellet was washed twice

with ice cold 66% ethanol in 0.1 M NaCl, and was then dissolved in 10 mL of water. The absorbance of the solution was determined at 260 and 280 nm. RNA concentration was estimated using the equation, 1 A<sub>260</sub> unit = 40 ug RNA (Maniatis, et al., 1982).

#### Binding of TPA to Cytochrome P-450

Microsomes were suspended in 50 mM Tris HCl (pH 7.4), containing 10% (v/v) of the nonionic detergent Tergitol NP-10, at a protein concentration of 1 mg / mL. The suspension was divided into reference and sample cuvettes, and a baseline difference spectrum of the oxidized cytochrome P-450 was measured over wavelengths 380-480 nm using a Perkin-Elmer 525 double beam spectrophotometer. TPA was added to a final concentration of 280 uM and a second difference spectrum recorded.

## In vitro Assay of TPA Effects on P-450 Levels

TPA (170  $\mu$ M) was added to an incubation system containing microsomes (2 mg protein/mL) and an NADPH regenerating system as described for the ethylmorphine demethylase assay. To a second group of flasks was added an equivalent volume of the dimethylsulfoxide carrier (TPA is stored as a stock solution of 50 mg/mL in dimethylsulfoxide). The mixtures were incubated in air for 30 minutes at 37 C, then were assayed for cytochrome P-450.

## Statistical Analysis

All data were analyzed by 2X2 ANOVA's according to Sokal and Rohlf (1969).

## RESULTS

### A. Effects of TPA on the Basal and Phenobarbital-Induced Hepatic Monooxygenase System in Intact Rats

#### i) Effects 24 hours after administration of TPA and/or phenobarbital

TPA (20 ug/kg) significantly reduced both basal and phenobarbital (110 mg/kg)-induced P-450 levels in intact male rats 24 hours post-treatment (Table 1). TPA reduced hepatic microsomal P-450 concentrations by approximately 27% ( $p < .025$ ) in rats not treated with phenobarbital, and by 23% in phenobarbital treated animals. A similar pattern was exhibited by aniline hydroxylase activity. TPA significantly lowered its basal values by 14%, and its phenobarbital-induced values by 5% (Table 2). Basal ethylmorphine demethylase activity was lowered 4%, and the phenobarbital-induced value, 3%, by TPA, but these decreases were not significant (Table 3). TPA lowered basal and phenobarbital-



TABLE 1. Effects of TPA (20 ug/kg, 24 hours prior to sacrifice, i.p.) and phenobarbital (110 mg/kg, 24 hours prior to sacrifice, i.p.) on the microsomal cytochrome P-450 content of male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.03 $\pm$ 0.12
TPA	0.84 $\pm$ 0.12 (p<.025)
PB	1.33 $\pm$ 0.04 (p<.001)
PB+TPA	1.15 $\pm$ 0.03

TABLE 2. Effects of TPA (20 ug/kg, 24 hours prior to sacrifice, i.p.) and phenobarbital (110 mg/kg, 24 hours prior to sacrifice, i.p.) on the activity of hepatic microsomal aniline hydroxylase in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Aniline Hydroxylation (nmol product/ mg MP/ min)
Control	1.20 $\pm$ 0.06
TPA	1.03 $\pm$ 0.09 (p<.05)
PB	1.35 $\pm$ 0.04 (p<.001)
PB+TPA	1.28 $\pm$ 0.02

TABLE 3. Effects of TPA (20 ug/kg, 24 hours prior to sacrifice, i.p.) and phenobarbital (110 mg/kg, 24 hours prior to sacrifice, i.p.) on the hepatic microsomal activity of ethylmorphine demethylase in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Ethylmorphine demethylation (nmol product./ mg MP/ min).
Control	9.14 $\pm$ 0.54
TPA	8.77 $\pm$ 0.75
PB	9.78 $\pm$ 0.47
PB+TPA	9.53 $\pm$ 0.43

induced activities of cytochrome c reductase by 10% and 26%, respectively, although again this effect was not statistically significant (Table 4). Thus, TPA decreased both basal and induced levels of cytochrome P-450 and the activity of aniline hydroxylase, but did not affect, statistically, at least, the other parameters measured, particularly ethylmorphine demethylase. Neither was a phenobarbital effect seen on this parameter. Over a longer time frame, at least, PB is known to strongly induce ethylmorphine demethylase (Rees, 1979). In order to test the possibility that this lack of effect by TPA on the demethylase was artifactual, we decided to increase the time of exposure to 48 hours.

ii) Effects 48 hours after administration of TPA  
and/or phenobarbital

When the rats were killed 48 hours after injection, the results were different from those obtained at 24 hours. TPA administration had no significant effects on basal levels or activities of the enzymes, although most did appear to be slightly depressed. Measurements of basal cytochrome P-450 levels and the activities of aniline hydroxylase and ethylmorphine demethylase were all lowered approximately 16%. TPA

TABLE 4. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and phenobarbital (110 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal cytochrome c reductase in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Cytochrome c Reduction (nmol reduced/ mg MP/ min)
Control	126 $\pm$ 17
TPA	113 $\pm$ 9
PB	169 $\pm$ 24
PB+TPA	134 $\pm$ 17

led to no apparent effect on basal ethylmorphine demethylase activity.

When coadministered with phenobarbital, TPA interacted synergistically ( $p < .001$ ) with the drug to enhance its induction of both aniline hydroxylase (Table 5) and cytochrome P-450 (Table 6). This synergism between the two compounds indicates that, in this time frame, phenobarbital acts differently in the presence than it does in the absence of TPA. No significant synergism of TPA and phenobarbital was seen for ethylmorphine demethylase (Table 7) or cytochrome c reductase (Table 8).

It is well documented that the activation of kinase C leads to the release of a number of pituitary and other hormones. Included in these are growth hormone (Summers, et al., 1985), ACTH (Abou-Samra, Catt, and Aguilera, 1986), thyroid stimulating hormone (Drust and Martin, 1985), various corticosteroids (Culty, Vilgrain, and Chambaz, 1984; Widmaier and Hall, 1985). All of these may affect, directly or indirectly, hepatic cytochrome P-450 levels (Rumbaugh, Kramer, and Colby, 1978). It is possible that the release of these hormones as elicited by TPA is affecting the system and masking the TPA effect seen at 24 hours. It is also possible that the hormonal milieu in the rats somehow affects the response to TPA in the presence of phenobarbital. Thus,

TABLE 5. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (110 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal aniline hydroxylase in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Aniline Hydroxylation (nmol product/ mg MP/ min)
Control	1.33 $\pm$ 0.07
TPA	1.11 $\pm$ 0.06
PB	1.53 $\pm$ 0.07 (p<.001)
PB+TPA	1.74 $\pm$ 0.04

PB X TPA Interaction: p<.001

TABLE 6. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (110 mg/kg, 48 hours prior to sacrifice) on the hepatic microsomal content of cytochrome P-450 in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.10 $\pm$ 0.07
TPA	0.92 $\pm$ 0.08
PB	1.57 $\pm$ 0.06 (p<.001)
PB+TPA	1.74 $\pm$ 0.04

PB X TPA Interaction: p<.005



TABLE 7. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (110 mg/kg, i.p., 48 hours prior to sacrifice) on the hepatic microsomal activity of ethylmorphine demethylase in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Ethylmorphine demethylation (nmol product / mg MP/ min)
Control	8.91 $\pm$ 1.2
TPA	8.55 $\pm$ 0.91
PB	11.8 $\pm$ 0.6 (p<.001)
PB+TPA	12.3 $\pm$ 0.5

TABLE 8. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (110 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal cytochrome c reductase in male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Cytochrome c reduction (nmol reduced/ mg MP/ min)
Control	126 $\pm$ 10
TPA	105 $\pm$ 6
PB	167 $\pm$ 20 (p<.025)
PB+TPA	175 $\pm$ 19

it was decided to repeat the experiments in hypophysectomized male rats in order to minimize the effects that the endocrine system might be exerting on the induction process.

#### B. Effects of TPA on Basal and Phenobarbital-

##### Induced Hepatic Parameters in Hypophysectomized Rats

##### i) Effects at 24 hours after administration of TPA and/or phenobarbital

Hypophysectomized rats are less able to metabolize phenobarbital than intact animals, and the dose administered to the latter (110 mg/kg) proved lethal to the hypophysectomized rats. Therefore, a lower dose (90 mg/kg) of phenobarbital was employed in experiments with hypophysectomized animals. The dose of TPA was unchanged at 20 ug/kg.

At 24 hours post-injection, it was found that, as in intact animals, TPA brought about general reductions in the levels of basal and phenobarbital-induced P-450 and associated activities. Basal cytochrome P-450 concentrations were lowered significantly 26%, by TPA, and the phenobarbital induced concentration of this hemoprotein was also

lowered 26% (Table 9). Since there was no statistical interaction between TPA and phenobarbital, it would appear that the two compounds are acting via the same mechanism. Basal ethylmorphine demethylase activity was significantly reduced 14% by TPA and the phenobarbital-induced activity was significantly decreased 21% by TPA (Table 10). TPA significantly lowered aniline hydroxylase activity by 30% and 13% in the basal and induced states, respectively (Table 11). Basal cytochrome c reductase activity was not affected by TPA (Table 12), but the phenobarbital-induced activity of this enzyme was 25% lower in TPA-treated animals compared to those treated only with phenobarbital, and, in fact, resembled the value obtained with untreated animals. At 24 hours, the effects of TPA in the hypophysectomized rat are qualitatively similar to those in intact rats but, are of greater magnitude. Moreover, TPA causes some significant depressant effects on basal and induced ethylmorphine demethylase and induced cytochrome c reductase activities that are not seen in intact rats. It appears that TPA is more effective in the hypophysectomized animal than in the intact. The additivity of the TPA and phenobarbital effects, indicated by the lack of statistical interaction between the two again suggests that they are acting via the same mechanism.

TABLE 9. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 24 hours prior to sacrifice) on the hepatic microsomal content of cytochrome P-450 in hypohysectomized male rats. Data are the means  $\pm$  S.E. for eight rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.41 $\pm$ 0.09
TPA	1.04 $\pm$ 0.11 (p<.001)
PB	1.81 $\pm$ 0.14 (p<.001)
PB+TPA	1.34 $\pm$ 0.20

TABLE 10. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 24 hours prior to sacrifice) on the hepatic microsomal activity of ethylmorphine demethylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for eight rats.

Treatment	Ethylmorphine demethylation (nmol product / mg MP/ min)
Control	4.06 $\pm$ 0.22
TPA	3.48 $\pm$ 0.31 (p<.001)
PB	5.95 $\pm$ 0.49 (p<.001)
PB+TPA	4.73 $\pm$ 0.77

TABLE 11. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal aniline hydroxylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for eight rats.

Treatment	Aniline Hydroxylation (nmol product/ mg MP/ min)
Control	0.648 $\pm$ 0.073
TPA	0.453 $\pm$ 0.031 (p<.001)
PB	0.815 $\pm$ 0.050 (p<.001)
PB+TPA	0.711 $\pm$ 0.056

TABLE 12. Effects of TPA (20 ug/kg/ i.p., 24 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal cytochrome c reductase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for eight rats.

Treatment	Cytochrome c Reduction (nmol reduced/ mg MP/ min)
Control	57.9 $\pm$ 3.8
TPA	57.7 $\pm$ 12.6
PB	87.2 $\pm$ 13.6 (p<.001)
PB+TPA	65.4 $\pm$ 4.0

PB X TPA Interaction: p<.05



ii) Effects at 48 hours after administration of TPA  
and/or phenobarbital

Because the results differ when obtained from intact rats treated with TPA and/or phenobarbital for 24 as opposed to 48 hours, it was of interest to determine whether or not a similar phenomenon occurred in hypophysectomized rats. It was found that the situation was different in these animals.

Basal ethylmorphine demethylase activity was significantly lowered, by 33%, relative to control by exposure to TPA for 48 hours (Table 13). Basal cytochrome P-450 was significantly lowered 34% by TPA (Table Fourteen), and basal aniline hydroxylase activity was significantly lowered by 32% (Table 15). Basal cytochrome c reductase activity was not significantly affected by TPA (Table 16).

TPA lowered phenobarbital-induced cytochrome P-450 levels by 39%. Ethylmorphine demethylase activity was not as strongly affected, being lowered by only 11% by TPA. Phenobarbital-induced aniline hydroxylase activity was lowered 16% by TPA and induced cytochrome c reductase activity was lowered, although not significantly, by 34%. The hypophysectomized animal differs markedly from its intact counterpart in this respect.

TABLE 13. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 48 hours prior to sacrifice), on the activity of hepatic microsomal ethylmorphine demethylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Ethylmorphine Demethylation (nmol product / mg MP/ min)
Control	4.83 $\pm$ 0.32
TPA	3.24 $\pm$ 0.40 (p<.05)
PB	8.16 $\pm$ 0.28 (p<.001)
PB+TPA	7.33 $\pm$ 0.40

TABLE 14. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 48 hours prior to sacrifice) on the hepatic microsomal content of cytochrome P-450 in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.14 $\pm$ 0.09
TPA	0.750 $\pm$ 0.029 (p<.05)
PB	2.01 $\pm$ 0.21 (p<.025)
PB+TPA	1.45 $\pm$ 0.22

TABLE 15. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal aniline hydroxylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Aniline Hydroxylation (nmol product/ mg MP/ min)
Control	0.722 $\pm$ 0.044
TPA	0.586 $\pm$ 0.053 (p<.05)
PB	1.08 $\pm$ 0.04 (p<.001)
PB+TPA	0.929 $\pm$ 0.048

TABLE 16. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal cytochrome c reductase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Cytochrome c Reduced
	(nmol reduced/ mg MP/ min)
Control	60.2 $\pm$ 5.5
TPA	56.1 $\pm$ 4.3
PB	105.3 $\pm$ 7.7 (p<.001)
PB+TPA	78.8 $\pm$ 6.4

Intact animals responded, after 48 hours exposure to TPA and phenobarbital with an enhancement in the induction of cytochrome P-450 and aniline hydroxylase activity.. This was unlike their cohorts exposed for only 24 hours, in which TPA lowered the phenobarbital-induced levels of these enzymes. The hypophysectomized animals, however, resembled their counterparts exposed for only 24 hours. The magnitude by which TPA lowered the phenobarbital-induced levels of the enzymes was the same at 48 hours as at 24 hours. Thus, the hypophysectomized animal differs from its intact counterpart in its response to phenobarbital. Hypophysectomized animals were employed in all subsequent experiments.

#### C.TPA Does Not Affect Apocytochrome P-450 Levels

Since we knew that TPA exerted a negative effect on the hepatic monooxygenase system (i.e., depressed total cytochrome P-450, and cytochrome c reductase), we next turned our attention towards the mechanism by which this might be effected. One explanation for our results might be that TPA somehow limits the availability of heme. Cytochrome P-450 consists of two moieties, the apocytochrome and heme. These are synthesized independently, so a deficiency in one can

limit the levels of the holocytochrome independently of the levels of the other. We therefore assayed the levels of the apocytochrome by the indirect method of Correia and Meyer (1975).

Rat liver homogenates were incubated with hemin as described in Materials and Methods. Microsomes were then prepared and total P-450 was then determined in the usual manner. As can be seen in Table 17, the holo-P-450 levels of the hemin treated microsomes did not differ from those incubated only with buffer. TPA does not apparently decrease P-450 levels by lowering the availability of heme to the apocytochrome. It is almost certain, therefore, that it is the amount of apocytochrome that is limiting. A deficiency in the degree to which the apocytochrome binds heme cannot be excluded, however.

#### D.TPA Does Not Directly Affect Cytochrome P-450

TPA might exert its negative effects on the hepatic mixed function oxidase system by directly binding to cytochrome P-450, thereby inactivating it. That this is not the case is supported by the results of the following experiments:

TABLE 17. Holocytochrome P-450 content in hepatic microsomes prepared from hypophysectomized male rats and incubated with hemin. The rats were treated with TPA (20 ug/kg, i.p., 24 hours prior to sacrifice), TPA + phenobarbital (90 mg/kg i.p., 24 hours prior to sacrifice), or TPA + 3-methylcholanthrene (25 mg/kg, i.p., 24 hours prior to sacrifice).

Treatment	Holocytochrome P-450 (nmol/ mg microsomal protein)	
	<u>-hemin</u>	<u>+hemin</u>
Vehicles	1.03±0.03	1.03±0.07
TPA	0.789±0.085	0.771±0.073
TPA+PB	1.39±0.16	1.32±0.11
TPA+3-MC	1.04±0.14	1.00±0.12



i) Lack of TPA binding to cytochrome P-450

Compounds which bind to the active site of cytochrome P-450 induce characteristic spectral changes due to their effects on the spin state of the heme iron (Sligar, 1976). That TPA does not bind to the active site of P-450 was evidenced by our finding that the addition of TPA (final concentration: up to 280  $\mu$ M) to detergent-solubilized microsomes containing 0.9  $\mu$ M cytochrome P-450 resulted in no spectral change over wavelengths 380-480 nm. (Figure Three). It is unlikely, therefore, that TPA decreases P-450 levels and associated activities by acting as an inhibitor or as a suicide substrate.

ii) Failure of TPA to destroy cytochrome P-450 in vitro

Microsomes and TPA (or the TPA vehicle, DMSO) were incubated in the presence of an NADPH-generating system for 30 minutes at 37 C as described in Materials and Methods. Total cytochrome P-450 was then measured. TPA concentration (170  $\mu$ M) was far in excess of that which could have existed in the liver in our in vivo experiments. Figure Four shows that TPA had no significant effect on the P-450 levels. The P-450 levels of both control and TPA-treated microsomes were

FIGURE THREE. TPA does not bind to cytochrome P-450. Detergent-solubilized microsomes containing 0.9  $\mu\text{M}$  cytochrome P-450 were scanned over wavelengths 380-480 nm. TPA was added to the sample cuvette to a concentration of 280  $\mu\text{M}$ , and the suspensions were rescanned over the same wavelengths. The upper tracing represents the microsomes in the TPA-containing cuvette minus the untreated microsomes of the reference cuvette. The lower tracing is the baseline untreated minus untreated microsomes.

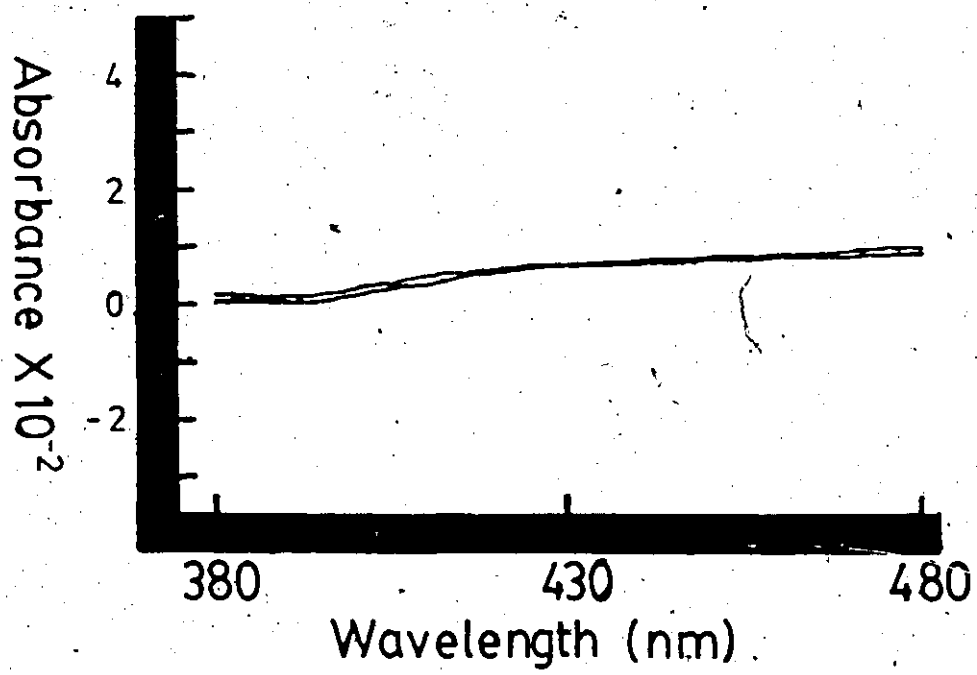
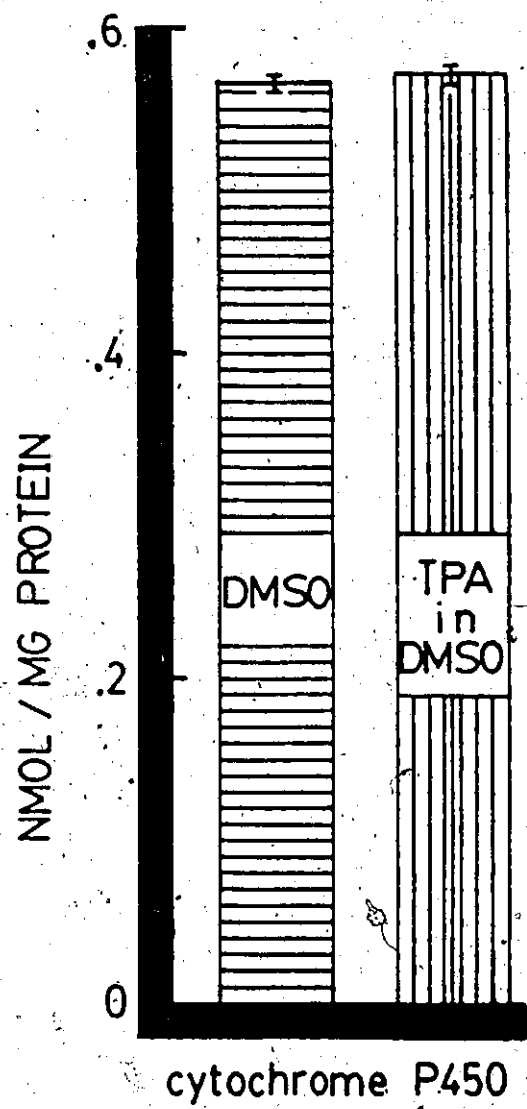


FIGURE FOUR. TPA does not directly destroy cytochrome P-450 in vitro. Microsomes (2 mg protein/mL) were incubated with 170  $\mu$ M TPA or the TPA vehicle, DMSO, for 30 minutes in the presence of an NADPH-generating system at 37C. Cytochrome P-450 was then assayed as described in Materials and Methods.



lower than normal ( $0.57 \pm 0.02$  and  $0.57 \pm 0.03$  nmols/mg microsomal protein, respectively) because of the DMSO. TPA does not directly destroy cytochrome P-450.

#### E. Inhibition of the TPA Effect<sup>o</sup> by Puromycin

Since TPA does not directly lower cytochrome P-450, its action must be mediated by other molecules. In an attempt to further characterize this mediation process, we inhibited protein synthesis with puromycin in animals treated with TPA. In so doing, the effects of any protein factors which TPA induces and which depress P-450 levels, or of any constitutive but short-lived mediator of the phorbol ester's effects would be lost. Indeed, the inhibitor eliminated the effects of TPA. TPA significantly lowered the activities of aniline hydroxylase (Table 18) and ethylmorphine demethylase (Table 19) as well as the levels of cytochrome P-450 (Table 20). Puromycin also decreased these parameters, but not significantly. When TPA and puromycin were administered together, the values obtained were indistinguishable from those where puromycin alone was administered. A significant interaction between the effects of TPA and those of puromycin were seen in all cases except for cytochrome c

TABLE 18. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and puromycin (50 mg/kg, i.p., 24 hours prior to sacrifice) on the hepatic microsomal activity of aniline hydroxylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Aniline Hydroxylation (nmol product/ mg MP/ min)
Control	0.735 $\pm$ 0.027
TPA	0.455 $\pm$ 0.017 (p<.001)
Puromycin	0.553 $\pm$ 0.013
Puromycin+TPA	0.550 $\pm$ 0.020

Puromycin X TPA Interaction: p<.001

TABLE 19. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and puromycin (50 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal ethylmorphine demethylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Ethylmorphine Demethylation (nmol product / mg MP/ min)
Control	5.34 $\pm$ 0.07
TPA	3.71 $\pm$ 0.14 (p<.025)
Puromycin	4.59 $\pm$ 0.26
Puromycin+TPA	4.53 $\pm$ 0.16

Puromycin X TPA Interaction: p<.025



TABLE 20. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and puromycin (50<sup>mg</sup>/kg, i.p., 24 hours prior to sacrifice) on hepatic microsomal cytochrome P-450 content in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.04 $\pm$ 0.03
TPA	0.681 $\pm$ 0.013 (p<.001)
Puromycin	0.791 $\pm$ 0.017
Puromycin+TPA	0.819 $\pm$ 0.038

Puromycin X TPA Interaction: p<.001

reductase, for which no effects whatsoever were seen (Table 21). The interaction between the two drugs indicated that TPA acts differently in the presence 50 mg/kg puromycin than in its absence. This is, of course, due to the fact that, while puromycin on its own has no significant effects, it eliminates the TPA effect. Puromycin did not significantly affect microsomal, cytosolic, or plasma protein levels, indicating that the inhibitor did not grossly affect the rats' health. These results obtained with puromycin support the hypothesis that either at least one component of the system mediating the TPA effect must be synthesized de novo upon TPA appearance, or is a short-lived protein.

#### F. TPA Effects on RNA Content of Liver

The next step in our investigation was to determine whether TPA might affect phenobarbital-mediated induction at the level of RNA. Phenobarbital is well known to increase cellular RNA, particularly ribosomal RNA (Rees, 1979). If TPA alters this increase in RNA, then its effects on the phenobarbital-initiated induction system may be at a pre-translational level.

RNA was isolated by homogenizing the liver in liquified

TABLE 21. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and puromycin (50 mg/kg, i.p., 24 hours prior to sacrifice) on hepatic microsomal cytochrome c reductase activity in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Cytochrome c Reduced
	(nmol reduced/ mg, MP/ min)
Control	45.1 $\pm$ 5.4
TPA	46.8 $\pm$ 2.1
Puromycin	49.3 $\pm$ 7.4
Puromycin+TPA	47.3 $\pm$ 4.3

phenol, followed by repeated extraction into distilled water as described by Kruh (1967). We found that after 24 hours of exposure to phenobarbital total RNA was increased by 48% (Table 22). TPA, itself, did not significantly alter RNA concentrations. However, TPA inhibited the inductive effect of phenobarbital and lowered its induction of RNA to only 34% over control. It is probable that TPA is interfering with the inductive effects of phenobarbital at the level of transcription.

#### G. Effects of TPA on 3-Methylcholanthrene-induced Hepatic Parameters

The results presented above provide evidence that TPA lowers hepatic P-450 levels and may even interfere with the increased transcription of the genes encoding them that is initiated by phenobarbital. What cannot be gleaned from these results is whether or not this interference is specific only to the phenobarbital-mediated inductive process or whether it is a more general phenomenon. To approach this problem, the effects of TPA on induction by a polycyclic aromatic hydrocarbon, 3-methylcholanthrene were studied.

Polycyclic aromatic hydrocarbons (PAH,s) induce a set

TABLE 22. Effects of TPA (20 ug/kg, 24 hours prior to sacrifice) and phenobarbital (90 mg/kg, i.p., 24 hours prior to sacrifice) on hepatic total RNA content in hypohysectomized male rats. Data are the means  $\pm$  S.E. for five rats.

Treatment	Total RNA (mg RNA/ g Liver)
Control	2.10 $\pm$ 0.07
TPA	2.47 $\pm$ 0.08
PB	3.10 $\pm$ 0.16 (p<.001)
PB+TPA	2.82 $\pm$ 0.14 (p<.05)

of cytochromes P-450 distinct from those induced by phenobarbital. The mechanism by which this induction occurs is apparently also different. A cytosolic receptor, analogous to those which mediate most actions of steroid hormones, binds the PAH, translocates to the nucleus, and activates the transcription of the genes encoding the cytochromes P-450 to be synthesized as well as those for other metabolic enzymes (Whitlock, 1986). There is no evidence that such a system is operational for phenobarbital. Therefore, if TPA acts to downregulate the P-450s induced by 3-methylcholanthrene as well as those induced by phenobarbital, then its effects must be considered to be of a more general nature.

When administered at a dose of 25 mg/kg, 3-methylcholanthrene significantly increased cytochrome P-450 by 27% (Table 23). TPA interfered with this induction, since in its presence the increase in P-450 levels was only 5.5%. By itself, TPA significantly lowered basal P-450 by 29%. The other parameters tested, i.e., ethylmorphine demethylase, aniline hydroxylase, and cytochrome c reductase, were not affected significantly by 3-methylcholanthrene (Tables 24-26). In intact animals, 3-methylcholanthrene normally lowers the activity of ethylmorphine demethylase presumably by diluting the isozymes supporting it with newly induced

TABLE 23. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and 3-methylcholanthrene (25 mg/kg, i.p., 24 hours prior to sacrifice) on hepatic microsomal cytochrome P-450 content in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.08 $\pm$ 0.05
TPA	0.827 $\pm$ 0.050 (p<.025)
3-MC	1.37 $\pm$ 0.03 (p<.01)
3-MC+TPA	1.14 $\pm$ 0.06

TABLE 24. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and 3-methylcholanthrene (25 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal ethylmorphine demethylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Ethylmorphine Demethylation (nmol product / mg MP/ min)
Control	3.93 $\pm$ 0.24
TPA	2.94 $\pm$ 0.30
3-MC	4.11 $\pm$ 0.35
3-MC+TPA	3.58 $\pm$ 0.35



TABLE 25. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and 3-methylcholanthrene (25 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal aniline hydroxylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Aniline Hydroxylation
	(nmol product/ mg MP/ min)
Control	0.727 $\pm$ 0.02
TPA	0.451 $\pm$ 0.05 (p<.05)
3-MC	0.777 $\pm$ 0.05
3-MC+TPA	0.662 $\pm$ 0.08

TABLE 26. Effects of TPA (20 ug/kg, i.p., 24 hours prior to sacrifice) and 3-methylcholanthrene (25 mg/kg, i.p., 24 hours prior to sacrifice) on the activity of hepatic microsomal cytochrome c reductase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for four rats.

Treatment	Cytochrome c Reduction (nmol reduced/ mg MP/ min)
Control	52.5 $\pm$ 6.5
TPA	45.7 $\pm$ 5.0
3-MC	59.9 $\pm$ 3.9
3-MC+TPA	46.4 $\pm$ 6.5

isozymes. The possibility must be considered, therefore, that induction was incomplete in these hypophysectomized rats. That P-450 levels were increased is, however, evidence that some sort of inductive event did occur.

It would appear that TPA is affecting processes general to the induction of several P-450 isozymes. It remains a possibility, however, that only a small subset of total P-450s is affected. The broader significance of the finding that TPA interferes with P-450 induction by 3-methylcholanthrene cannot be determined without a knowledge of the isozymes affected by TPA.

#### H.TPA and Chlorpromazine

Finally, it was decided that the effects of TPA on induction by chlorpromazine, another reported phenobarbital-like hepatic P-450 inducer should be tested. This drug has also been reported to inhibit the activation of kinase C (Mori, et al., 1980). If the hypothesis that phenobarbital exerts at least some of its inductive effects through an inhibitory effect on kinase C is true, then the effects of TPA on induction by this kinase C inhibitor should be similar to those seen with phenobarbital.

TABLE 27. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and chlorpromazine (12 mg/kg, i.p., 48 hours prior to sacrifice) on the hepatic microsomal content of cytochrome P-450 in hypophysectomized male rats. Data are the means  $\pm$  S.E. for six rats.

Treatment	Cytochrome P-450 (nmol/ mg microsomal protein)
Control	1.26 $\pm$ 0.09
TPA	0.909 $\pm$ 0.041 (p<.05)
CPZ	1.01 $\pm$ 0.11
CPZ+TPA	1.00 $\pm$ 0.06

TABLE 28. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and chlorpromazine (12 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal ethylmorphine demethylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for six rats.

Treatment	Ethylmorphine Demethylation (nmol product / mg MP/ min)
Control	4.23 $\pm$ 0.18
TPA	3.68 $\pm$ 0.09
CPZ	3.35 $\pm$ 0.45
CPZ+TPA	3.41 $\pm$ 0.30

TABLE 29. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and chlorpromazine (12 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal aniline hydroxylase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for six rats.

Treatment	Aniline Hydroxylation (nmol product/ mg MP/ min)
Control	0.692 $\pm$ 0.036
TPA	0.486 $\pm$ 0.038 (p<.025)
CPZ	0.598 $\pm$ 0.048
CPZ+TPA	0.544 $\pm$ 0.027

TABLE 30. Effects of TPA (20 ug/kg, i.p., 48 hours prior to sacrifice) and chlorpromazine (12 mg/kg, i.p., 48 hours prior to sacrifice) on the activity of hepatic microsomal cytochrome c reductase in hypophysectomized male rats. Data are the means  $\pm$  S.E. for six rats.

Treatment	Cytochrome c Reduced
	(nmol reduced/ mg MP/ min)
Control	42.3 $\pm$ 2.7
TPA	36.2 $\pm$ 3.9
CPZ	35.5 $\pm$ 4.5
CPZ+TPA	30.9 $\pm$ 3.9

Unfortunately, as can be seen in Tables 27 through 30, this drug failed, when administered at a dose of 12 mg/kg, to induce the hepatic systems tested. This dose has been effective at inducing P-450 in intact rats (Kato and Vassanelli, 1962). In fact, chlorpromazine appeared to decrease both cytochrome P-450 levels (Table 27) and ethylmorphine demethylase activity (Table 28), albeit, insignificantly. Thus, we do not know what effect TPA might have had should induction have occurred.

#### Summary of Results

1. TPA (20ug/kg), when administered intraperitoneally, lowered both basal and phenobarbital-induced hepatic P-450 levels in intact male rats when assayed 24 hours post-injection. Basal and induced aniline hydroxylase activities were similarly affected. However, neither basal nor induced activities of ethylmorphine demethylase or cytochrome c reductase were significantly lowered by TPA.

2. TPA lowered basal P-450 levels and aniline hydroxylase and cytochrome c reductase activities in intact



males at 48 hours post-injection, and increased the phenobarbital-induced levels of P-450 and activity of aniline hydroxylase.

3. In hypophysectomized male rats, 24 hours post-injection, TPA lowered basal values of cytochrome P-450, ethylmorphine demethylase, and aniline hydroxylase, but had no effect on cytochrome c reductase. TPA inhibited the induction of all four parameters by phenobarbital.

4. In the hypophysectomized male, the effects of TPA at 48 hours post-injection resembled those at 24 hours, both in the presence and absence of phenobarbital. This suggests that in the hypophysectomized rat, complications seen in its intact counterparts are absent. In the intact rat, the effects of TPA may be complicated by the hormonal milieu.

5. TPA administration does not result in limitations of heme availability to apocytochrome P-450. Reconstitution experiments showed no excess of apocytochrome P-450 as indicated by a failure to produce higher quantities of spectrally-assayed holocytochrome through preincubation with hemin. It is, therefore, likely that apocytochrome synthesis is lowered by TPA.

6.TPA does not destroy cytochrome P-450 directly. When microsomes were incubated with high concentrations of TPA, no effect on P-450 levels was seen.

7.TPA does not bind to cytochrome P-450. Even at a concentration of 280  $\mu$ M, there were no spectral changes over wavelengths 380-480 nm in detergent solubilized microsomes containing 0.9 nmoles P-450 / mg microsomal protein.

8.Puromycin blocks the inhibitory properties of TPA. The values obtained for all parameters in the presence of both TPA and puromycin are indistinguishable from those obtained with puromycin alone.

9.TPA has no significant effect on hepatic total RNA but attenuates the increase in total RNA promoted by phenobarbital. It is likely, therefore, that TPA interferes with phenobarbital-mediated induction at a pretranslational level.

10.TPA lowers 3-methylcholanthrene-induced levels of cytochrome P-450. Thus, it appears that the effect of TPA on P-450 induction may be general, applying to several classes of inducer. Without a knowledge of the

specific isozymes affected by TPA, a final determination of the generality, or lack thereof, cannot be made.

11. Chlorpromazine failed to induce cytochrome P-450.

## DISCUSSION

There are two major phenobarbital-inducible isozymes of hepatic cytochrome P-450, P-450b and P-450e, each encoded by distinct genes (Whitlock, 1986). The transcription of these genes is greatly increased by phenobarbital (Adesnick, et al., 1981; Atchison and Adesnik, 1983). Transcription of P-450b, for example, is increased 20 to 50 fold by phenobarbital (Hardwick, Gonzalez, and Kasper, 1983b; Pike, et al., 1985). Yet the mechanism by which this transcription is increased remains unknown. The fact that the rate of transcription begins to increase within one hour of phenobarbital administration (Hardwick, Gonzalez, and Kasper, 1983b) suggests that there are few intermediary steps in this induction process.

In addition to increasing the rate of transcription, phenobarbital increases the stability of mRNA (Venkatesan and Steele, 1975), and rRNA (Smith, et al., 1977). The effect of the latter is to increase intracellular rRNA levels by as much as 40% (Rees, 1979). Phenobarbital also decreases the turnover of hepatic proteins (Cajone and Bernelli-Zazzera, 1984). Essentially nothing is known about the mechanisms by which any of these effects are mediated. Another effect of phenobarbital is to increase the rate of translation, again by an unknown

mechanism. It would seem reasonable to postulate that phenobarbital increases translation by affecting the rate limiting step in this process, which is under phosphorylation/ dephosphorylation control (Farrell, Hunt, and Jackson, 1978; Pain, 1986).

Phosphorylation/dephosphorylation is a common means of metabolic control. Such a mechanism is employed in the regulation of the levels and activities of the steroidogenic cytochromes P-450 of the adrenal cortex and gonads. This regulation is best understood in the adrenal cortex.

The primary physiological regulator of adrenocortical P-450 expression is the pituitary hormone corticotropin (CTH). This hormone binds to a specific receptor on the cell surface. The activated receptor then activates adenylate cyclase on the inner surface of the plasma membrane. Adenylate cyclase converts ATP to 3',5'-cyclic AMP (cAMP) and pyrophosphate. The cAMP thus produced then activates cyclic AMP-dependent protein kinase (protein kinase A). This activation leads, via phosphorylation events, to both an immediate increase in the rate of steroid synthesis, and to an increase in the levels of the steroidogenic enzymes themselves (Boggaram, Zuber, and Waterman, 1984b; Funkenstein, et al., 1983; John, et al., 1985; Zuber, Simpson, and Waterman, 1985). These steroidogenic enzymes

include four cytochromes P-450. Thus, it is through the activation of a protein kinase that the synthesis of adrenocortical cytochromes P-450 is induced. For a review of this process, see Simpson and Waterman (1983).

A mechanism analogous to that employed in the adrenal cortex has been suggested for the phenobarbital-mediated induction of hepatic monooxygenases. In 1976, Byus, et al., reported that after the administration of phenobarbital, the activation of cyclic AMP-dependent protein kinase preceded induction of ethylmorphine demethylase. Nothing has since been published with regard to this finding. We have found that TPA, a potent activator of protein kinase C, affects cytochrome P-450 expression. It is likely that protein kinase C is directly involved in the regulation of hepatic cytochromes P-450, and it is likely that the kinase has a role in the phenobarbital-mediated induction of these hemoproteins.

It has been known for some time that kinase C affects the activities of cytochromes P-450 in organs other than the liver. For example, in the adrenocortical zona glomerulosa, promotion of kinase C activity by TPA results in increased steroidogenesis (Kojima, et al., 1983, Kojima, et al., 1984). Although it was not directly tested, this increased steroidogenesis was probably due, at least in part, to the

induction of the necessary cytochromes P-450. Activation of adrenocortical steroidogenesis is known to involve an increased transcription of the genes encoding products necessary for that steroidogenesis (Boggaran, Simpson, and Waterman, 1984; John, et al., 1985; Zuber, et al., 1985).

In the liver, our work has shown that TPA interferes with the phenobarbital-mediated induction of cytochrome P-450. This interference has been shown to be unrelated to any direct effect of the phorbol ester on these enzymes. Rather, based on our finding that TPA impedes the increase of hepatic RNA promoted by phenobarbital, it seems likely that TPA interferes with the phenobarbital-induced changes in transcriptional activity.

If TPA activates hepatic kinase C, and the activated kinase then depresses the transcription of P-450 encoding-genes, then a means must exist to transfer the information of kinase C activation to the genes. The system is likely to be complex as some genes are repressed by the kinase, while others are activated. Moreover, the kinase is highly likely to affect the function of a large number of proteins not involved in the transcriptional regulation of P-450 genes. Some effects of the kinase are likely to appear later than others. Phenobarbital's effects are also complex, and to some extent, less than concerted (Adesnik and

Atchison, 1986).

The ultimate effector of the TPA effect on hepatic P-450 is highly likely to be protein kinase C. However, our finding that puromycin completely negates the TPA effect suggests that some other peptide is also involved in mediating TPA's effect. In cultured adrenocortical cells, at least, the activation of kinase C by TPA has been reported to be unaffected after 60 minutes exposure to high doses of puromycin or cycloheximide, another inhibitor of translation (Widmaier and Hall, 1985). If this is also true in the liver, over a longer term, then puromycin must be preventing the synthesis of a molecule involved in regulating hepatic cytochromes P-450 and cytochrome c reductase.

Angel, et al. (1987) have recently described a cis element common to genes inducible by phorbol esters. This is an eight base pair element located 72 to 185 base pairs upstream of the translational start. The element is the binding site for transcription factor AP-1, a recently discovered activator of transcription (Angel, et al., 1987)). Treatment of cultured cells with TPA rapidly leads to a 3- to 4-fold increase in AP-1 binding to this site, suggesting that the factor is the molecule responsible for effecting the kinase C-initiated activation of gene-specific transcription.



Since our work indicates that the activation of kinase C lowers the expression of the phenobarbital-inducible cytochromes P-450; it seems unlikely that AP-1 is directly involved in the regulation of the genes encoding them. Furthermore, the gene for phenobarbital-inducible P-450b contains two sequences similar to the consensus sequence for AP-1 binding (at positions -72 and -65) (see Suwa, et al., 1985), but both lack highly conserved nucleotides and are, therefore, unlikely to be true AP-1 binding sites. Since P-450 expression seems to be decreased rather than increased by phorbol esters, one would not expect to find such a domain in these genes. AP-1 is unlikely to be directly involved in hepatic P-450 gene downregulation.

If TPA effects on hepatic P-450 gene expression are not mediated through AP-1, then how might they be effected? How might phenobarbital affect this process? From our data, we know that TPA decreases basal cytochrome P-450, interferes with the induction of this hemoprotein by phenobarbital, and prevents the induction by phenobarbital of cytochrome c reductase. We know also, from this work, that TPA interferes with the induction of RNA by phenobarbital. A role for kinase C in phenobarbital-mediated induction does seem likely.

A simple model consistent with the data would postulate that TPA acts to promote the kinase C dependent

phosphorylation of a molecule which, when phosphorylated, would bind to negative elements in the regulatory regions of phenobarbital-inducible genes. In this way, transcription would be inhibited. Basal kinase C activity would be sufficient, under normal physiological conditions, to accomplish this inhibition. In this model, phenobarbital would act to inhibit the basal activity of the kinase so that the inducible genes would be released from negative control. They would thus be transcribed.

How might phenobarbital interfere with kinase C activity? Chauhaun and Brockerhoff (1987) have produced evidence that phenobarbital directly inhibits the activation of kinase C by diacylglycerol. It does this by competing with the diacylglycerol for the activating site on the enzyme. That phenobarbital would inhibit kinase C activation is consistent with our own findings of antagonism between TPA and phenobarbital in affecting hepatic enzymes. TPA and phenobarbital seem to act by the same mechanism, as evidenced by the statistical additivity of their effects. If activation of kinase C lowers hepatic P-450 levels and associated activities, and if phenobarbital inhibits kinase C, then it seems reasonable to argue that kinase C is a phenobarbital receptor.

If kinase C is the receptor for phenobarbital, then

another recent report deserves consideration. As mentioned in the Introduction, Ohno, et al. (1987), have found that all kinase C genes encode a "zinc finger" domain in the kinase. Also present in glucocorticoid- and estradiol- receptors, zinc fingers are DNA binding domains. Since phenobarbital binds to kinase C, and kinase C has at least the potential to bind DNA, then perhaps kinase C could be a receptor analogous to that of the polycyclic aromatic hydrocarbons. Kinase C, when bound to phenobarbital, might translocate to the nucleus and activate specific genes. This is highly speculative, and is probably less likely than kinase C acting through the synthesis of an intermediary protein. It is of interest, nevertheless, to note that the glucocorticoid receptor may also be a protein kinase (Singh and Moudgil, 1984).

All of this is interesting, but any model for the role of kinase C in hepatic P-450 regulation must account for all of the effects of the kinase in this regard. TPA, in addition to impeding induction by phenobarbital, impedes the induction of P-450s by the polycyclic aromatic hydrocarbon, 3-methylcholanthrene. The cytochromes P-450 induced by 3-methylcholanthrene are distinct from those induced by phenobarbital and are encoded on another chromosome (Whitlock, 1986). Furthermore, their induction is

known to be via a mechanism distinct from that of the phenobarbital-inducible isozymes. That TPA interferes with induction by 3-methylcholanthrene supports the view of a more general role for kinase C in cytochrome P-450 regulation. This by no means, however, eliminates the possibility that it is kinase C that serves as the receptor for phenobarbital.

It has been noted in the past that the treatment of hepatoma cells with phenobarbital leads to the induction of both phenobarbital and 3-methylcholanthrene-inducible isozymes of cytochrome P-450 (Wiebel, Kiefer, and Mardia, 1984; Wiebel, et al., 1984). The same phenomenon has been noted in the livers of fetal rats at term (Kremers, et al., 1981). Perhaps some aspects of the regulation of phenobarbital- and 3-methylcholanthrene-inducible genes are common to both. Secondary regulatory systems might mask these common features as development progresses. Evidence of a sort of coordinate regulation of the two P-450 classes in adulthood does exist.

While phenobarbital does not affect the levels of 3-methylcholanthrene-inducible cytochromes P-450 (Adesnick and Atchison, 1986), treatment of adult rats with 3-methylcholanthrene not only lowers the basal levels of phenobarbital-inducible P-450s (Yeowell, et al., 1985), but also prevents phenobarbital-type inducers from inducing these--

isozymes (Dannan, et al., 1983), apparently by preventing the transcription of the genes encoding them (Yeowell, et al., 1985). Activation of 3-methylcholanthrene-inducible genes prevents the activation of those inducible by phenobarbital. Another activator of the 3-methylcholanthrene-inducible genes, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been reported to activate kinase C (Bombick, et al., 1985) although not by a direct mechanism (Kramer, Sando, and Holsapple, 1986). Perhaps this is a signal through which the activation is impeded. Certainly, there is evidence for the existence of a short-lived molecule which negatively regulates the genes encoding 3-methylcholanthrene inducible P-450 isozymes.

The induction of cytochromes P-450 by 3-methylcholanthrene or other polycyclic aromatic hydrocarbons can be significantly enhanced, in cell culture, at least, by cotreatment with a translational inhibitor such as cycloheximide (Whitlock and Gelboin, 1973). This super-induction includes "superincreased" levels of cytochrome P-450 mRNA (Israel and Whitlock, 1983), a result of "superincreased" transcription of the genes encoding them (Israel, et al., 1985). It would certainly appear that there exists a short-lived protein that negatively regulates the expression of these P-450s at the level of transcription.

From our experiments with puromycin, we know that whatever mediates the negative effects of TPA is short-lived and/or induced by the drug.

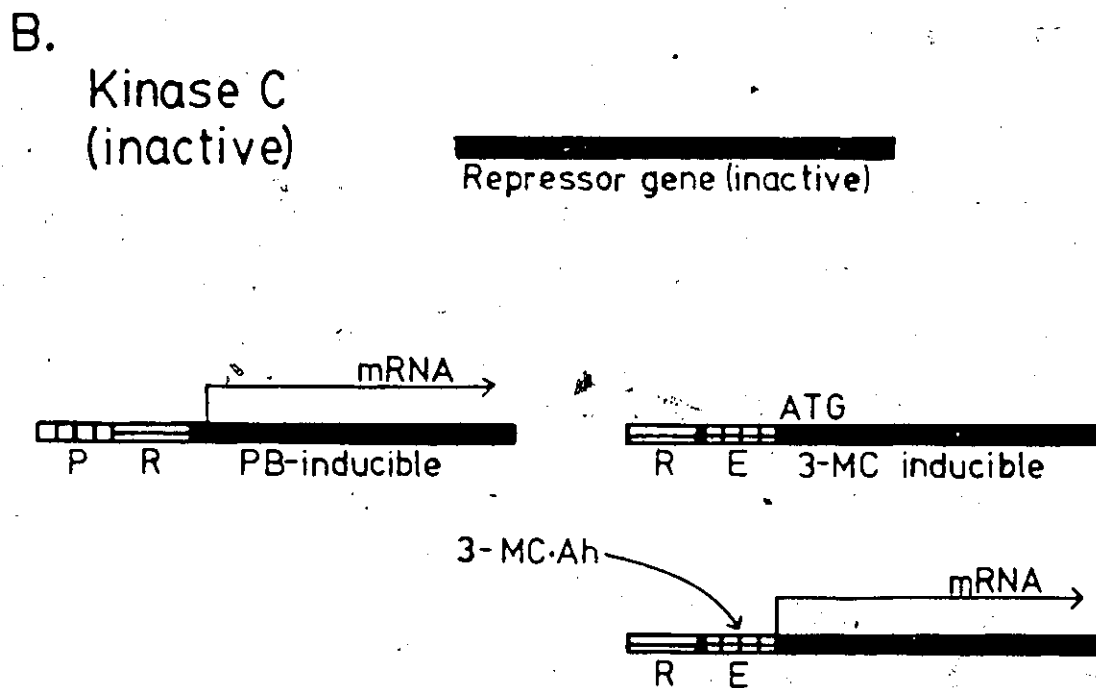
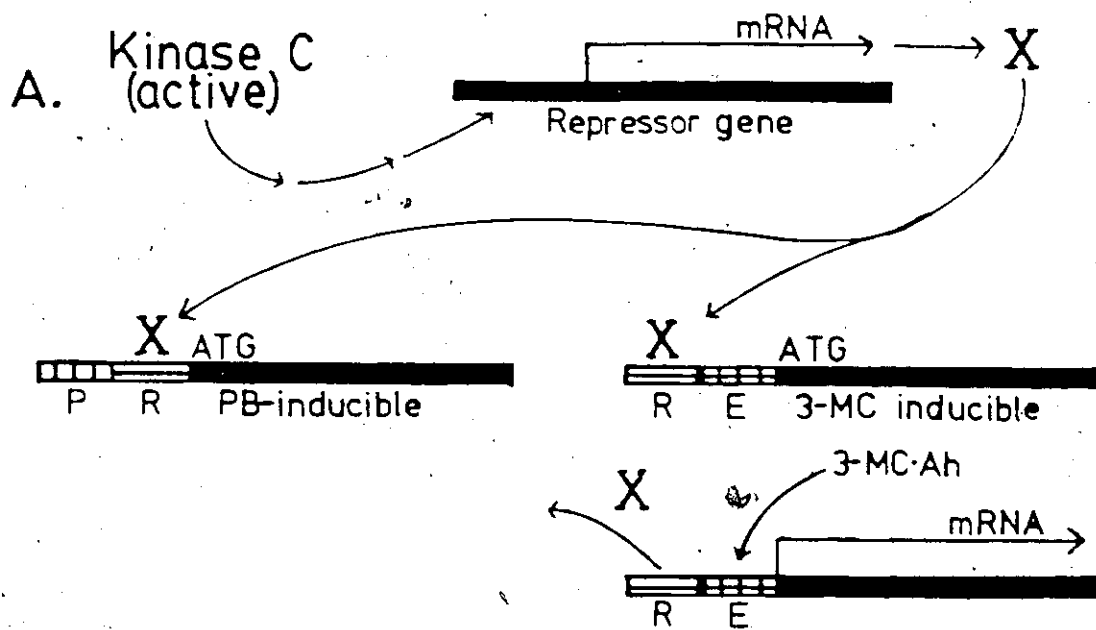
Figure Five illustrates a model for P-450 regulation which can be used to account for our findings and those from the literature discussed above. In this model, there exists a labile repressor molecule which impedes the transcription of both phenobarbital- and 3-methylcholanthrene-inducible genes. The levels of this repressor are under the transcriptional control of protein kinase C.

When kinase C is active, even at basal levels, the levels of the repressor are high. Pharmacological activation of the kinase elevates repressor levels even higher. When kinase C activity is lowered by a molecule such as phenobarbital, transcription of the repressor-encoding gene is reduced, and, since the repressor is labile, repressor levels fall rapidly. Transcription of the phenobarbital-inducible genes then begins.

A phenobarbital-inducible gene, under this model, would be expected to contain a fairly strong promoter, which, when released from the negative control of the repressor, would promote the efficient transcription of the coding region. Genes inducible by 3-methylcholanthrene, however, would not have such a constitutive strong promoter, and would require

FIGURE FIVE. A model for the regulation of phenobarbital (PB) and 3-methylcholanthrene (3-MC) inducible genes.

- A. Under normal physiological conditions, kinase C activity causes transcription of a repressor gene whose product (X) binds to regulatory elements (R) in both PB- and 3-MC-inducible genes, preventing their transcription. Ah receptor bound to acceptor sites (E) in the 3-MC-inducible genes overcomes the repressor effect on those genes.
- B. Kinase C activity is inhibited by PB, preventing repressor gene activity, and X is lost. In the absence of the X, the phenobarbital-inducible genes, which contain a strong promoter (P), are actively transcribed. 3-MC-inducible genes contain no such promoter and are not transcribed.





the well characterized enhancer activity associated with polycyclic aromatic hydrocarbon receptor-binding for increased transcriptional rates. Because of this, phenobarbital, by lowering repressor levels, would increase transcription of only phenobarbital-inducible genes.

3-Methylcholanthrene-inducible genes would require their own inducer in order to be transcribed. When 3-methylcholanthrene or another polycyclic aromatic hydrocarbon was present, kinase C would be activated, increasing repressor levels and preventing the transcription of the phenobarbital-inducible genes, even if phenobarbital was present. Conceivably, the polycyclic aromatic hydrocarbon receptor might act directly to increase repressor gene transcription. The ability of phenobarbital to increase the transcription of

3-methylcholanthrene-inducible genes in the neonate and in cell culture might be related to some constitutive promoter activity associated with these genes that is repressed as differentiation progresses.

If the model as presented is correct, then some means must exist whereby 3-methylcholanthrene-inducible genes can be transcribed in spite of the presence of the repressor. This could be accomplished in a number of ways. The binding sites for both the repressor and the polycyclic aromatic hydrocarbon receptor could overlap. Or, the binding of the

PAH receptor to its acceptor sites on the DNA could evoke changes in the secondary structure of the DNA (eg., B  $\rightarrow$  Z) such that the repressor can no longer readily bind. In other words, binding of the PAH receptor would effectively displace the repressor and activate the gene. The repressor could still bind occasionally, however, limiting the extent of induction. Inhibition of protein synthesis would result in loss of this labile repressor and allow the superinduction that has been seen under precisely these conditions. One would also expect superinduction of phenobarbital-inducible P-450 RNAs under these conditions if the model is correct. Unfortunately, to my knowledge, this has never been tested.

The model described herein would account for much that has been observed with regard to the induction of hepatic cytochromes P-450. It provides a potential explanation for phenobarbital-mediated induction and the relationship of that induction to that mediated by 3-methylcholanthrene. A number of other possibilities obviously exist, but the model presented here is simple and testable. Confirmation would require considerable work at the molecular level. In any case, the model ties together a significant number of observations previously unconnected.

In summary, we have found that the kinase C activator, TPA, both lowers basal hepatic cytochrome P-450 levels and

associated activities, and impedes their induction by phenobarbital and 3-methylcholanthrene. As TPA impedes the induction of RNA by phenobarbital, and has no direct effect on cytochrome P-450, it is likely that the phorbol ester exerts its effects on these genes at a level prior to translation, most likely at transcription. Puromycin blocks the TPA effect. Therefore a mediator of the TPA effect must either be induced by the TPA or is labile. It seems likely that kinase C is involved in the regulation of cytochromes P-450 in the liver of the rat.

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